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In Drosophila melanogaster, a 65 amino acid protein called Reaper is central regulator of apoptosis. Transcriptional induction of reaper is necessary for programmed cell death in flies and reaper deletion prevents apoptosis. Although no vertebrate homologs have been identified, our lab discovered that recombinant Reaper induces rapid apoptosis when added to Xenopus egg extracts. In the extract, Reaper causes cytochrome c release from the mitochondria into the cytosol where it acts a cofactor for the activation of "death proteases," or caspases. Using a Reaper affinity column, our lab purified a Reaper interacting protein named Scythe. Scythe is highly conserved among vertebrates and necessary for Reaper induced apoptosis in the Xenopus egg extract system. The goal of this proposal was to elucidate the mechanism of Reaper/Scythe action. Toward this end I established assays to measure apoptosis induced by ectopic Reaper expression in cultured human cells, expressed recombinant Reaper and Scythe in human tissue culture cells, and purified a truncated human Scythe protein produced in bacteria. Research progress with respect to the Statement of Work is detailed below.

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Introduction

In Drosophila melanogaster, a 65 amino acid protein called Reaper is a central regulator of apoptotic cell death. Transcriptional induction of Reaper precedes programmed cell deaths in flies, and Reaper deletion prevents these deaths. Furthermore, ectopic expression of Reaper promotes rapid apoptosis. Despite intensive effort, no vertebrate Reaper homologs have been described. Our lab reported several years ago that recombinant Drosophila Reaper protein induces rapid apoptosis upon addition to Xenopus egg extracts. This cell death results primarily from Reaper-induced release of cytochrome c from mitochondria to the cytosol; as in many other apoptotic pathways, cytochrome c thus released serves as a cofactor for activation of "death proteases," or caspases. Using a Reaper affinity column, Ken Thress in our lab purified a 150kD Reaper-interacting protein from Xenopus egg extracts, which he named Scythe. Scythe is highly conserved in vertebrates. Immunodepletion of Scythe from egg extracts prevented Reaper-induced in vitro apoptosis. Moreover, a truncated Scythe variant induced apoptosis independently. The goal of this proposal was to elucidate the mechanism of Scythe/Reaper action.

Body of Report:

This grant was originally awarded as a predoctoral fellowship to Ken Thress in our laboratory. When Ken graduated, I was given the opportunity to assume the remainder of the award. Ken has already submitted a final report on his part of the fellowship in which he accomplished most, if not all, of Technical Objective II. This work resulted in the publication of two papers in EMBO J. that demonstrated that Scythe is a suppressor of Hsc 70-mediated protein folding and that this suppression can be reversed by Reaper. More recent work in the laboratory has demonstrated that Reaper/Scythe and Hsc 70 can act on mitochondrial proteins to promote cytochrome c release. This work is more extensively documented in the report of Michael Olson, another USAMRC-funded pre-doctoral fellow in the lab. I have been funded by this grant for approximately one year. However, in this time, I have accomplished much of the proposed work in Technical Objective I. During this time I played a key role in establishing mammalian cell culture assays in the Kornbluth lab, where most of the previous work in the lab had been done in vitro in the Xenopus egg extract system. This work has opened up many new avenues in the lab, which I will be pursuing in the next two years towards completion of my PhD. The following accomplishments correspond to the original objectives as outlined in the statement of work:

Task 1: To achieve desired expression of Scythe and Reaper in mammalian tissue culture cells. Towards this end, I subcloned Reaper into a number of mammalian expression vectors for the production of myc-tagged, FLAG-tagged and untagged Reaper. Scythe was expressed as a myc-tagged protein in a

pcDNA3 backbone, as proposed. See Fig. 1, appended for documentation of expression of these proteins in mammalian cultured cells.

Task 2: Establishment of apoptotic assays to determine the effects of Reaper and Scythe in mammalian cells. Working with another student in the lab, Chris Holley, I found that the proposed TUNEL and FACS analyses were not as useful to us as direct caspase assays and direct counting of GFP co-transfectants surviving after Reaper transfection. Cell death by Reaper in mammalian cells is documented in Fig. 2. In addition, we found that overexpression of Scythe could act as in Xenopus to at least partially suppress Reaper-induced cell death. We are currently producing a human Scythe antibody because we suspect that Scythe may, like its relative Bag-1, be overexpressed in tumors where it might act to suppress endogenous apoptotic signals.

Task 3: Cloning of a C-terminal activated fragment of human Scythe (also called BAT3). Dr. Thress had established that a corresponding fragment from Xenopus Scythe could induce apoptosis autonomously. We have produced the human form of this protein recombinantly and are currently working to produce it in mammalian cells to determine if it, too, will autonomously induce apoptosis. See Fig. 3, appended for documentation of production of the C-terminal activated fragment of human Scythe.

Tasks 4 and 5 are underway.

Key research accomplishments:

- Establishment of mammalian apoptotic assays for Reaper
- Construction of recombinant truncated human Scythe and insertion into mammalian expression vectors
- Establishment of vectors for the production of Reaper in mammalian cell cultures.

Reportable outcomes:

Although in the short time I was funded by the grant I was not able to complete a publishable unit of work, my role in the establishment of mammalian apoptosis assays in the lab allowed me to participate in another ongoing project, resulting in my co-authorship of the following publication:

Smith, J.J., Richardson, D.A., Kopf, J., Yoshida, M., Hollingsworth, R.E., and Kornbluth, S. (2002). Apoptotic Regulation by the Crk adapter protein mediated by interactions with Wee1 and Crm1/Exportin. Mol. Cell. Biol. 22: 1412-1423.

Conclusions:

The long term goal of the original proposal was to elucidate the mechanism of action of Reaper/Scythe. The original work of Dr. Thress established the involvement of Scythe-mediated Hsc-70 inhibition as part of the apoptotic mechanism. Coupled with my work setting up the mammalian tissue culture assays, this groundwork leaves us poised to fully elucidate the mechanism of this interesting protein complex.

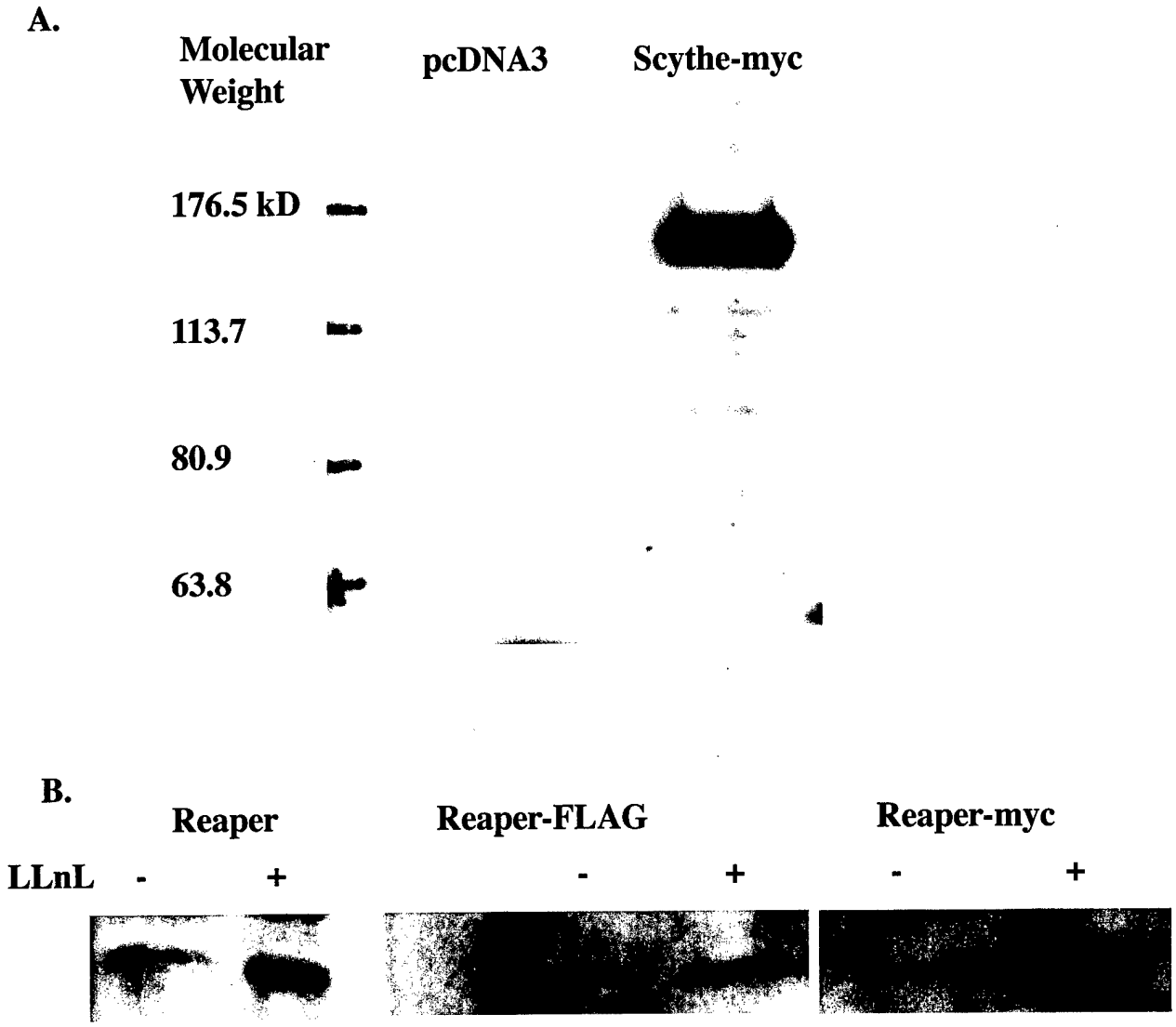

Figure 1: Expression of Scythe and Reaper in Cultured Human Cells
(A.) Expression of myc tagged Scythe in 293T cells. One million cells persample were transfected according to standard protocols. Cells were lysed, and thelysates were cleared by centrifugation at 14,000 rpm for five minutes. Myc-Scythe was immunoprecipitated using myc anti-sera, eluted with SDS sample buffer, and run on 7.5% SDS-PAGE, transfered to PVDF membranes, and immunoblotted with anti-myc antibodies. (B) Reaper expression in 293T cells. Untagged or tagged Reaper constructs were expressed in 293T cells and processed as described in (A). Indicated samples were mock treated or exposed to 20 μM LLnL for 45 minutes prior to lysis to enhance Reaper stability. Reaper was immunoblotted with antibodies raised against Reaper, FLAG, amd myc respectively.

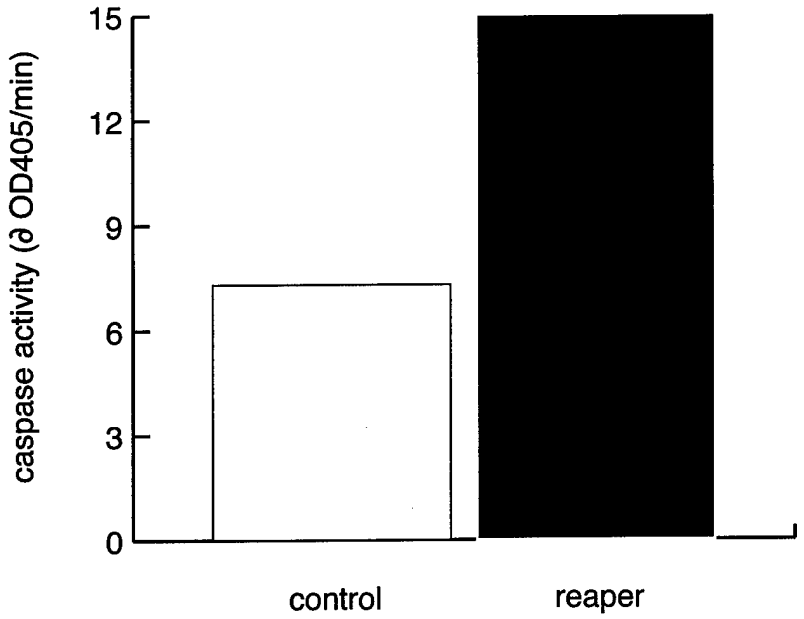

Figure 2: Reaper mediated caspase activation in cultured human cells.
$1x10^6$ 293T cells were transfected by standard methods with vectors indicated. After 24h, adherent and floating cells were collected and lysed in 100 μL of caspase lysis buffer. Cells were incubated on ice 10 min then centrifuged 10 min to remove debris. The caspase assay was prepared with 50 μL of cell supernatent plus 10 μL of colorimetric caspase-3 substrate and 40 μL of assay buffer. Reactions were allowed to proceed at 37 degrees Celsius and substrate cleavage was measured at OD 405nm. Results are expressed as rates of caspase-3 substrate cleavage.

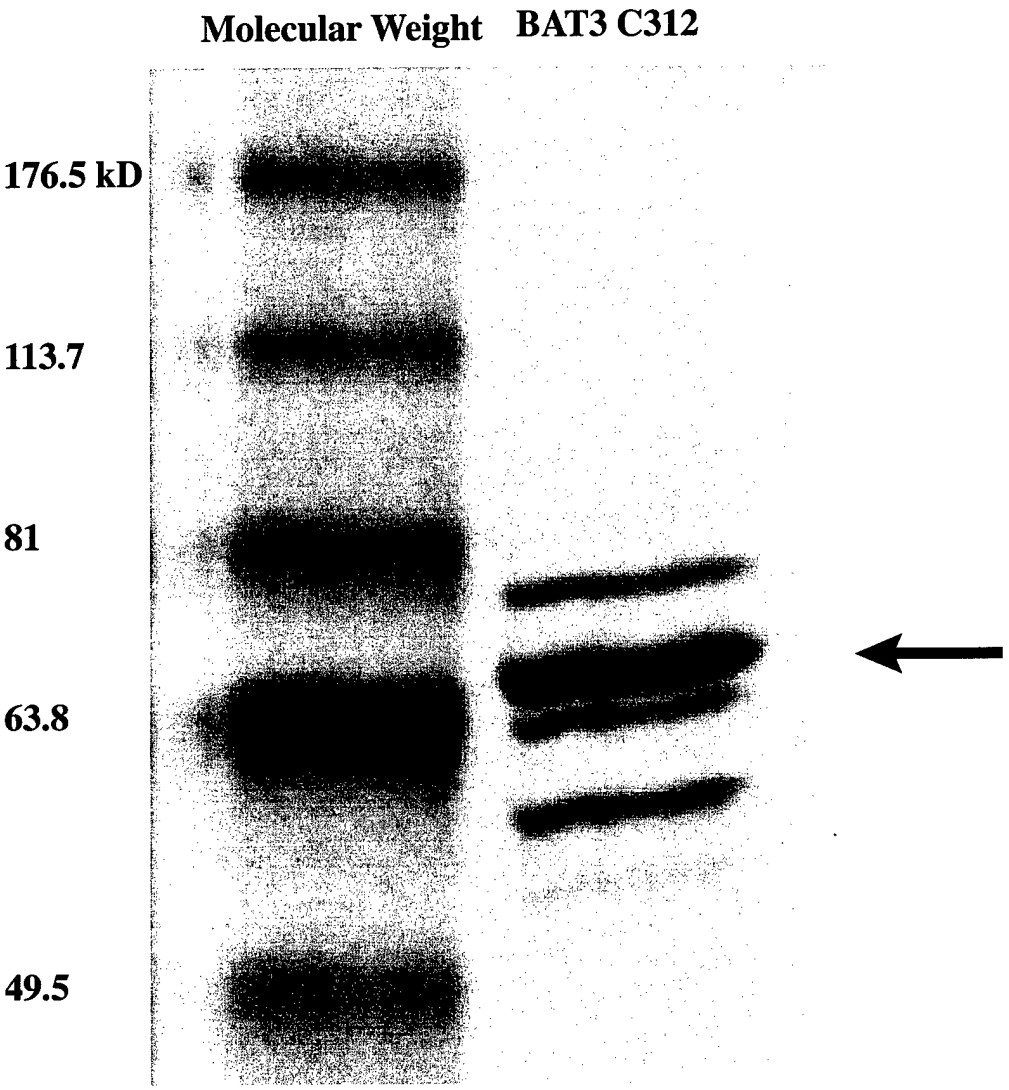

Figure 3: Recombinant expression of human Scythe C-terminal activated fragment (BAT3 C312) The BAT3 C312 encoding sequence was subcloned into the pGEX-KG vector for expression as a GST fusion protein. This plasmid was transformed into the TOPP1 *E. coli* strain, grown to mid-log density, induced using 1 mM IPTG at 37 degrees celsius for three hours, and purified on glutathione agarose beads according to standard protocols. The BAT3 C312 major band is indicated by the arrow.

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Apoptotic Regulation by the Crk Adapter Protein Mediated by Interactions with Wee1 and Crm1/Exportin

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The adapter protein Crk contains an SH2 domain and two SH3 domains. Through binding of particular ligands to the SH2 domain and the N-terminal SH3 domain, Crk has been implicated in a number of signaling processes, including regulation of cell growth, cell motility, and apoptosis. We report here that the C-terminal SH3 domain, never shown to bind any specific signaling molecules, contains a binding site for the nuclear export factor Crm1. We find that a mutant Crk protein, deficient in Crm1 binding, promotes apoptosis. Moreover, this nuclear export sequence mutant [NES(−) Crk] interacts strongly, through its SH2 domain, with the nuclear tyrosine kinase, Wee1. Collectively, these data suggest that a nuclear population of Crk bound to Wee1 promotes apoptotic death of mammalian cells.

In many different intracellular signaling pathways, information is transmitted through protein-protein interactions mediated by the modular protein binding domains SH2 and SH3 (for review, see references 2 and 37). These domains, which bind tyrosine-phosphorylated proteins and polyproline-rich motifs, respectively, can be contained either within large enzymes, such as c-Abl, where they may regulate catalytic activity, substrate selection, and interaction with upstream regulators, or within small adapter proteins, such Crk, Nck, and Grb2, which contain no intrinsic enzymatic activity.

The predominant paradigm for adapter protein signaling involves localization of adapter-bound SH3 ligands to specific subcellular locales via interaction of the SH2 domain of the adapter with specific tyrosine-phosphorylated proteins. This is exemplified by the localization of the Ras GTP exchange factor, Sos, to the plasma membrane following ligand engagement of receptor tyrosine kinases (e.g., epidermal growth factor receptor [EGFR]) (4). Through binding to tyrosine-phosphorylated residues on the intracellular domain of the receptor, the adapter protein Grb2 brings SH3-bound Sos to the membrane, where it can activate Ras (5, 7, 40).

That inappropriate adapter protein signaling can have severe consequences for the cell was first suggested by the observation that a protein with homology to the viral oncoprotein Src, but lacking any obvious catalytic domain, could promote oncogenic transformation (29, 48). This protein, v-Crk, encoded by the avian sarcoma virus CT10, contains the viral Gag protein fused to sequences encoding an SH2 domain and an SH3 domain. Two cellular homologs of this protein, Crk I and Crk II, have since been shown to consist of one SH2 domain and either one or two SH3 domains, respectively (28, 38; for review, see references 14 and 27). The Crk II protein, containing two SH3 domains, is at least 10-fold more abundant than Crk I in most tissues, and the linker region between the Crk II SH3 domains contains a site of potential tyrosine phosphorylation, believed to serve as a site of regulatory intramolecular SH2 binding (10, 13, 38). Finally, a close relative of Crk (CrkL) has been identified that has overall structural similarity and high sequence homology to Crk II (33, 34, 46).

Since Crk lacks intrinsic catalytic activity, a good deal of effort has gone into identifying binding partners for its SH domains and determining the physiological contexts in which they act. Crk has been linked to cell proliferation through its SH2-mediated interactions with tyrosine-phosphorylated Cbl, Shc, and EGFR (1, 6, 26; for review, see reference 14). More recently, it has become clear that Crk plays a role in cell adhesion signaling and actin reorganization through Crk recruitment of SH3-bound Dock 180 (a regulator of the GTPase Rac) to tyrosine-phosphorylated p130Cas, found at focal adhesions and sites of membrane ruffling (8, 9, 19, 20, 22, 23). Additionally, using cell extracts prepared from *Xenopus* eggs, we have previously implicated Crk in apoptotic signaling (12, 42).

Although *Xenopus* egg extracts are best known for their use in reconstituting cell cycle progression and nuclear trafficking, more recently it was shown that these extracts can be used to examine the morphological and biochemical events of apoptosis (11, 12, 24, 25, 32, 42, 47). As is the case in most intact mammalian cells, apoptosis in these extracts is characterized by activation of apoptotic proteases (caspases), release of cytochrome *c* from the intermembrane space of the mitochondria to the cytosol (where it serves as a cofactor in caspase 9 activation), activation of DNases, and concomitant fragmentation of nuclei. Importantly, these hallmarks of apoptosis, which appear after extended incubation of the extract at room temperature, can be prevented by common inhibitors of apoptosis, such as ZVAD, YVAD, and DEVD (caspase inhibitors), and anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-xL (11, 24, 25, 32).

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When we analyzed the requirements for apoptosis in *Xenopus* extracts, we found that the adapter protein Crk was absolutely required for mitochondrial cytochrome *c* release and consequent caspase activation (12). Indeed, immunodepletion of endogenous Crk protein or addition of anti-Crk sera to the extracts completely abrogated apoptotic signaling. Perhaps most surprising was our finding that the Crk SH2 ligand important for proapoptotic signal transmission in these extracts was the known Cdc2/cyclin B inhibitor Wee1 (42). In a series of biochemical experiments, we demonstrated that Wee1, like Crk, is required for apoptotic activation of *Xenopus* egg extracts. Furthermore, Wee1's proapoptotic function depends upon its interaction with Crk. Because chemical inhibitors of Cdc2 as well as the Wee1-related Cdc2/cyclin regulator Myt1, did not exhibit apoptotic effects similar to that of Wee1, we hypothesized that the role of Wee1 in apoptosis is distinct from its cell cycle regulatory role and involves signaling via the Crk adapter protein (42).

We report here the finding that mammalian Crk II is similar to its *Xenopus* counterpart in its ability to bind tyrosine-phosphorylated Wee1. In searching for other potential components of this complex, we also made the surprising discovery that the second SH3 domain of Crk II, which, unlike the first SH3 domain, has never been shown to bind any signaling molecule, interacts with the nuclear export receptor, Crm1. We have identified a candidate Crm1-binding NES within the second (C-terminal) SH3 domain and show that ablation of this sequence both enhances Wee1-Crk binding and increases the proapoptotic activity of the Crk protein. These findings implicate a nuclear pool of Crk-Wee1 complexes in signaling pathways that promote apoptosis.

MATERIALS AND METHODS

Plasmids and primers. pCDNA 3.0 myc-Wee1 was graciously provided by Helen Piwnica-Worms (Department of Cell Biology and Physiology, Washington University, School of Medicine, St. Louis, Mo.). pCNDA 3.0-caspase-8 was the generous gift of Vishva Dixit (Department of Molecular Oncology, Genentech, Inc., San Francisco, Calif.). Glutathione *S*-transferase (GST) fusions of the Crk SH2 domain, SH3(N) domain, and SH3(C) domain constructs have been described previously (12). The GST fusion of the cyclin B1 NES (or cytoplasmic retention sequence) has been described previously (52). Wild-type Crk II cloned into the vector pEBB (pEBB-wt Crk II) has been described previously (45) and was provided by Bruce J. Mayer (Department of Genetics and Developmental Biology, University of Connecticut Health Center, Farmington). NES(−) Crk cloned into pEBB was generated by overlap extension PCR (21). The PCR product [NES(−) Crk] was cloned into pEBB by using *Bam*HI and *Not*I sites at the 5′ and 3′ ends, respectively. The mutagenesis primers used for this procedure are as follows. The inner primers were 5′-TTGGAGGTCGGTGAGGCGGCA AAGGCCACGAAGATTAACATGAGT-3′ and 5′-ACTCATGTTAATCTTC GTGGCCTTTGCCGCCTCACCGACCTCCAA-3′. The outer primers were 5′-GGATCCACCATGGCCGGGCAG-3′ and 5′GCGGCCGCTCAGCTGAAG TC-3′.

pEBB-myc-wt Crk and pEBB-myc-NES(−) Crk were created by inserting a three-myc tag at the 5′ end of the Crk constructs by using the *Bam*HI site. The GST-NES(−) Crk SH3(C) construct was prepared by PCR amplification of the SH3(C) domain of pEBB NES(−) Crk. The PCR product was cloned into pGex kg (a derivative of pGex 2T; Pharmacia) by using *Bam*HI and *Hin*dIII sites at the 5′ and 3′ ends, respectively. The following primers were used for this process: 5′-GGATCCTTGGCTTTGGAGGTCGGTGAG-3′ and 5′-AAGCTTTCATTG ATCCAGCAGGCGGACATG-3′.

For expression of GST-wt Crk and GST-NES(−) Crk in bacteria, the respective cDNAs were excised from pEBB by using the *Bam*HI and *Xba*I (5′ and 3′ ends, respectively) and subsequently cloned into pGex kg. Expression and purification of GST fusion proteins in bacteria were performed according to the method of Evans et al. (12).

Yeast two-hybrid assay. The cDNA insert from pEBB-wt Crk II was PCR amplified and cloned into the DNA binding domain fusion vector PMW101, which has been described previously (51). The yeast two-hybrid screening protocol was performed as has been described previously (41). The Crk II bait was screened versus one million clones derived from human fetal brain, human fetal liver, and human testis prey libraries (purchased from InVitrogen, Inc.).

Antibodies. An anti-myc monoclonal antibody (clone 9E10) was purchased from Santa Cruz. Anti-FLAG monoclonal antibody was obtained from Sigma. Anti-phosphotyrosine monoclonal antibody (clone 4G10) was purchased from Upstate Biotechnology. Anti-Crk monoclonal antibody was purchased from Transduction Laboratories. Anti-Crk polyclonal antisera was generously provided by Bruce J. Mayer. Anti-Crm1 polyclonal immunoglobulin G (IgG) was provided by Gerard Grosveld (Department of Genetics, St. Jude Children's Research Hospital, Memphis, Tenn.). For immunofluorescence, the secondary antibody, a goat anti-mouse–fluorescein isothiocyanate (FITC) conjugate, was purchased from Jackson Immunological, Inc.

Preparation of *Xenopus* egg extracts. Preparation of crude interphase egg extracts has been described previously (12). To fractionate the crude egg extract into cytosolic and membranous components, the crude extract was centrifuged further at 55,000 rpm (200,000 × *g*) in a Beckman TLS-55 rotor for the TL-100 centrifuge for 1 h. The cytosolic fraction was removed and recentrifuged for an additional 25 min at 200,000 × *g*. The light membrane fraction was removed and diluted into 1.5 ml of egg lysis buffer (ELB) (250 mM sucrose, 2.5 mM $MgCl_2$, 1 mM dithiothreitol [DTT], 50 mM KCl, 10 mM HEPES [pH 7.7]) and subsequently pelleted through a 0.5 M sucrose cushion at 20,000 rpm for 20 min. The heavy membrane fraction (enriched in mitochondria) was removed, and the mitochondrial fraction was purified further by centrifugation of the heavy membrane through a Percoll gradient consisting of 42, 37, 30, and 25% Percoll in mitochondrial isolation buffer (1 M sucrose, 100 mM ADP, 2.5 M KCl, 1 M DTT, 1 M succinate, 1 M HEPES-KOH [pH 7.5], 0.5 M EGTA, 1.5 M mannitol) for 25 min at 25,000 rpm with no brake in the TLS-55 rotor. The isolated heavy membrane fraction containing mitochondria was diluted 1:10 into ELB containing an ATP-regenerating cocktail (20 mM phosphocreatine, 2 mM ATP, 5 μg of creatine phosphokinase per ml).

Reconstituted extracts were then generated from these extract fractions with purified mitochondria added to cytosol at ratios ranging from 1:20 to 1:40 (mitiochondria-cytosol). To extracts, in which nuclei were formed, light membrane was added to the mixture of cytosol and mitochondria at a ratio of 1:10. Nuclei were formed in these extracts by addition of demembranated sperm chromatin (~2,000 nuclei/μl). These reconstituted extracts were supplemented with an energy-regenerating system consisting of 2 mM ATP, 5 μg of creatine kinase per ml, and 20 mM phosphocreatine (final concentrations). Recombinant proteins added to these extracts were diluted into XB buffer (protein concentrating buffer; 100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM KOH-HEPES [pH 7.7], 50 mM sucrose) at the indicated protein concentrations.

DEVDase assays. To assay caspase activity, 3-μl aliquots of each extract sample (at various time points) were incubated with 90 μl of DEVDase buffer (50 mM HEPES [pH 7.5], 100 mM NaCl, 0.1% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 10 mM DTT, 1 mM EDTA, 10% glycerol) and the colorimetric peptide substrate Ac-DEVD-pNA (200 mM, final concentration; BIOMOL Research Labs, Inc.). Enzyme reaction mixtures were incubated at 37°C for 30. The absorbance of the colorimetric product was measured at 405 nm with a LabSystems MultiSkan MS mictrotiter plate reader.

CSH3 binding protein pulldown assays. The GST, Crk SH3(C), Crk SH3(C)-NES-deficient, Crk SH3(N), and B1CRS recombinant proteins were linked to glutathione-Sepharose beads. For pull-down assays analyzed by Western blotting, 50 μl of beads was incubated with 300 μl of extract for 1 h at 4°C. The beads were then pelleted and washed three to five times with ELB. The bound proteins were eluted in 2× SDS sample buffer and separated on SDS-polyacrylamide gel electrophoresis (PAGE) gel (7.5% polyacrylamide).

Cell culture and transfection. U2OS, Cos-7, and HEK 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM [Cellgro]; 4.5 g of glucose per liter, with L-glutamine) supplemented with 10% fetal calf serum (Sigma). NIH 3T3 cells were maintained in DMEM (4.5 g of glucose per liter, with L-glutamine) with 10% bovine calf serum. U2OS, Cos-7, and 293 cells were transfected with FuGene 6 (Roche) transfection reagent (4 μl). 3T3 cells were transfected with Lipofectamine (GIBCO-BRL; 20 μl per 35-mm-diameter dish, 100 μl per 100-mm-diameter dish) according to the manufacturer's protocol.

Tissue culture cell apoptosis assays. Apoptosis induced by overexpression of Crk constructs was monitored by using a luciferase assay as described previously (30). Modifications to this procedure were as follows. 293 or Cos-7 cells were plated (1.5×10^5 to 2.5×10^5 cells per 35-mm-diameter well) in six-well dishes and transfected with 1.5 μg of pEBB, pEBB-wt Crk, pEBB-NES(−) Crk, or

pCNDA 3.0 caspase-8. Along with each gene of interest, the constitutively active luciferase reporter construct PGL3 (Promega) was cotransfected (DNA amount, 1:10). After 24 to 48 h of transfection, cells were collected and washed twice with phosphate-buffered saline (PBS). Cells were lysed in six-well dishes with 250 μl of lysis buffer (1% Triton X-100, 25 mM glycyl glycine [pH 7.8], 4 mM EGTA, 1 mM DTT). Lysates were clarified by centrifugation at 16,000 × *g* for 10 min. Lysate samples (100 μl) were then mixed with 275 μl of ATP solution (20 mM $MgSO_4$, 5 mM ATP, 0.7 mg of bovine serum albumin [BSA] per ml, 25 mM glycyl glycine [pH 7.8]). Each sample was subsequently mixed with 100 μl of 1 mM luciferin (ICN) in 25 mM glycyl glycine. Light emission was measured with a Berthold Lumat luminometer model no. LB 9501. The relative ratio of cell death was calculated as the inverse of relative luciferase activity (arbitrary units).

Apoptotic morphologies were confirmed by plating cells on coverslips and transfection with wild-type and NES(−) Crk constructs. DNA was stained with Hoechst 33258 dye, and characteristic apoptotic nuclear morphologies were scored. Additionally, relative rates of apoptosis were determined as follows:

pEBB wild-type and NES(−) Crk constructs were individually cotransfected into 293, NIH 3T3, and Cos-7 cells (seeded on coverslips) with a green fluorescent protein (GFP) reporter construct (pEGFP N2; Clontech; DNA amount, 1:10). Coverslips were collected 24 h posttransfection and washed twice in PBS. Cells were then fixed with 4% paraformaldehyde in PBS for 10 min. Relative rates of apoptotic killing were calculated by scoring the percent of surviving GFP cells per total number of cells in a field. For each transfectant, six fields (×20 air objective) containing at least 200 cells were analyzed.

IP from mammalian tissue culture cells. Immune resins were generated by incubation of 5 μg of anti-myc or anti-FLAG (negative control) monoclonal antibodies, with 30 μl of protein G-Sepharose (Oncogene), or 20 μg of anti-Crk antisera or preimmune sera (negative control) with 30 μl of protein A-Sepharose (Amersham). Antibodies were incubated with protein A (or G)-Sepharose for 1 h at 4°C in 200 μl of immunoprecipitation (IP) buffer supplemented with 1% BSA. Before incubation with cell lysates, the resin was pelleted by centrifugation, and the supernatant was removed.

To generate cell lysates, 100-mm-diameter tissue culture dishes were seeded with NIH 3T3, Cos-7, or 293 cells that were grown to 50 to 70% confluency. Sixteen to 20 h posttransfection, medium was removed from cells, and the cells were washed twice with PBS. Cells were lysed in 500 μl of IP buffer (50 mM HEPES [pH 7.4], 1 mM EDTA, 1% NP-40, 150 mM NaCl, 1 mM DTT) supplemented with 0.2 mM sodium orthovanadate and aprotinin-leupeptin (5 μg/ml, final concentration). Cell lysates were sonicated by six rapid bursts from a Branson Sonifier 150 on output setting 4, and the insoluble material was pelleted by centrifugation at 16,000 × *g* for 10 min at 4°C. Protein concentrations of lysates were determined by using the Bio-Rad protein assay dye such that they could be normalized by dilution in IP buffer.

Cell lysates (~500 μg of total protein per sample) were incubated with immune resins for 1 h at 4°C with gentle agitation. The resins with bound proteins were pelleted by brief centrifugation, and the lysates were removed by aspiration. Subsequently, immune resins were washed four times in IP buffer. Bound proteins were eluted by boiling in SDS-PAGE sample buffer and resolved by SDS-PAGE, with one-half of the total immunoprecipitated material loaded on the gel.

Immunoblotting. Immunoblotting was performed following SDS-PAGE and transfer to polyvinylidene difluoride (PVDF) membranes (Millipore). Blots were incubated with appropriate primary antisera described above and subsequently (secondary antibodies) with horseradish peroxidase-linked protein A (Amersham) or goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, Inc.). Blots were developed with an enhanced chemiluminescence kit (Renaissance; Dupont NEN).

Crk immunostaining. U2OS cells were seeded on sterile glass coverslips (3.5×10^4 to 5.0×10^4 cells per coverslip) and allowed to attach overnight in DMEM (Cellgro; 4.5 g of glucose per liter, with L-glutamine) supplemented with 10% fetal bovine serum (Sigma). Following 16 to 24 h of transfection, coverslips were washed twice in 1 ml of PBS. Cells were fixed in 4% paraformaldehyde in PBS for 10 min. Coverslips were subsequently washed twice in PBS and then permeabilized in 0.2% Triton X-100 in PBS for 10 min on ice. Coverslips were rinsed again twice in PBS and incubated in block solution (3% BSA and 0.02% Triton X-100 in PBS) overnight at 4°C. Coverslips were then incubated for 30 min with primary antibody (anti-myc monoclonal) diluted 1:500 in block buffer. Cells were rinsed three times in wash buffer (1% BSA and 0.02% Triton X-100 in PBS) for 5 min with gentle agitation. Subsequently, coverslips were incubated in secondary antibody (goat anti-mouse–FITC) diluted into block buffer at 1:200 for 30 min. Excess antibody was removed by washing coverslips three times for 5 min each in wash buffer. The final wash was supplemented with Hoechst 33258 (1 μg/ml) to stain nuclear DNA. Coverslips were mounted on slides by using Anti-Fade mounting medium (Molecular Probes). Crk staining and nuclear DNA staining were visualized by fluorescence microscopy.

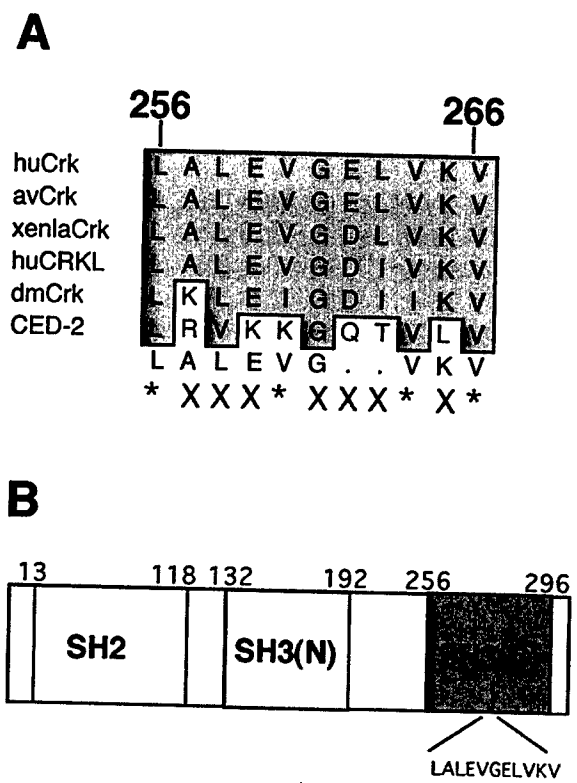

FIG. 1. Crk II contains a putative NES in its SH3(C) domain. The Crm1-binding consensus sequence, or NES, is defined by Bogerd et al. (3) (for the human T-cell leukemia virus type 1 protein Rex) as L–X_{2-3}–{F, I, L, V, M}–X_{2-3}–L–X–L. This definition has been expanded to include substitutions of valine or isoleucine for leucine (e.g., see reference 52). (A) Displayed here is an alignment of the putative NES from an array of metazoan Crk homologues: human Crk II (huCrk), avian Crk II (avCrk), *Xenopus laevis* Crk II (xenlaCrk), human Crk-like protein (huCRKL), *Drosophila melanogaster* Crk II (dmCrk), and *C. elegans* Crk II (CED-2). (B) Map of the Crk Src homology (SH) domains within its primary amino acid sequence: SH2 domain (residues 13 to 118), N-terminal SH3 domain [SH3(N); residues 132 to 192], and C-terminal SH3 domain [SH3(C); residues 256 to 296]. Note the putative NES is highlighted within the SH3(C) domain.

RESULTS

The C-terminal SH3 domain of Crk interacts with the nuclear export factor, Crm1. While our previous work had demonstrated that the SH2 domain of Crk could engage tyrosine-phosphorylated Wee1, we wished to identify and characterize additional proteins that might participate in apoptotic signaling through binding to either of the two SH3 domains. In an effort to isolate such molecules, we performed a yeast two-hybrid screen of human fetal brain, human fetal liver, and human testis cDNA libraries by using the full-length Crk II as bait. From a screen of one million prey clones, the only strongly positive Crk interactor to emerge was the human nuclear export protein, Crm1 (15, 35). This protein, a member of the importin family of nuclear transport receptors, mediates the export of its substrates from the nucleus to the cytoplasm in a Ran-GTP-dependent manner. Because Crm1 binds to a

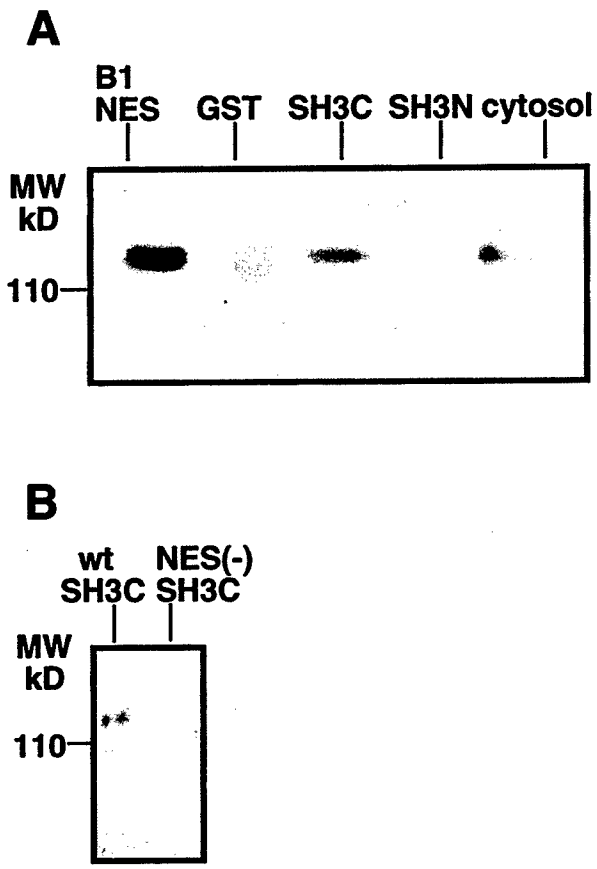

FIG. 2. Crm1 binds the C-terminal SH3 of Crk. (A) Equivalent levels of GST fusions of Crk SH3(N), Crk SH3(C), cyclin B1 NES (B1NES), and GST alone were bound to glutathione-Sepharose resin. Resins were incubated with egg extracts in order to precipitate specific binding proteins. Nonspecifically associated material was removed by washing in ELB three times. Bound proteins were eluted by boiling in SDS-PAGE sample buffer and detected by immunoblotting with a polyclonal anti-Crm1 anti-sera. Positive controls for Crm1 binding include cyclin B1 NES resin and egg extract cytosol (40 μg). Negative controls for Crm1 binding include Crk SH3(N) and GST. (B) The same protocol was performed with a GST fusion of the mutated Crk SH3(C) domain [NES(−) SH3(C)] and controls similar to those listed above. MW, molecular mass (kilodaltons).

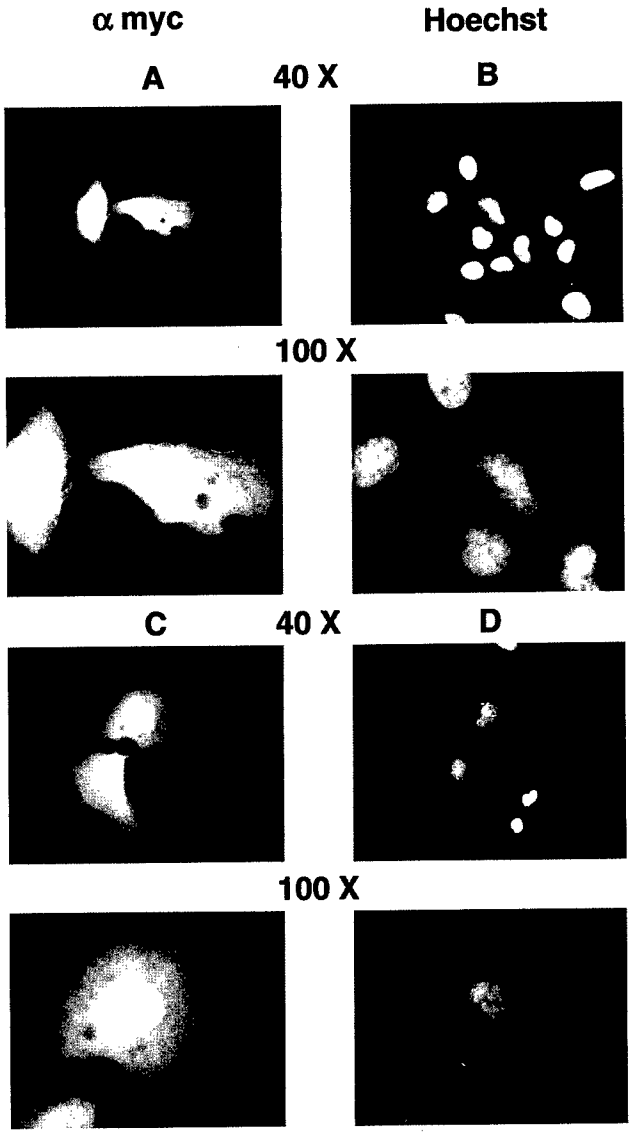

FIG. 3. U20S cells, seeded on glass coverslips, were transiently transfected with 1.0 μg of either pEBB myc-wt Crk (A and B) or pEBB myc-NES(−) Crk (C and D). Coverslips were processed for immunostaining with an anti-myc monoclonal antibody followed by secondary staining with a goat anti-mouse–FITC secondary antibody as described in Materials and Methods. Panels A and C display transfected Crk immunostaining, while panels B and D show Hoechst 33258 staining of nuclear DNA corresponding to panels A and C, respectively.

well-characterized, albeit loose, consensus sequence (3), we scanned the primary amino acid sequence of Crk for a similar motif that might serve as an NES (Fig. 1A). In doing so, we identified a putative NES lying within the C-terminal SH3 domain [SH3(C)] (Fig. 1B).

To confirm the interaction between Crm1 and the C-terminal SH3 domain of Crk, we prepared a resin composed of the GST-CrkSH3(C) linked to glutathione-Sepharose. Subsequently, this resin was incubated with *Xenopus* egg extract, pelleted, and washed extensively, and bound proteins were resolved by SDS-PAGE for immunoblotting with anti-Crm1 sera. As shown in Fig. 2A, precipitates formed with either the SH3(C) resin or a control NES resin (derived from the NES of cyclin B1) contained Crm1 (115 kDa), while control precipitates performed with a GST resin did not. The N-terminal SH3 domain, which lacks any observable NES-like sequence, was also unable to bind Crm1. In order to confirm that the putative NES sequence identified in Crk SH3(C) was, in fact, responsible for the observed Crm1 binding, we produced a mutant SH3(C) resin in which several residues predicted to be critical for NES function were changed to Ala (residues 263, 264, and 266). This mutant form of the SH3(C), NES(−) SH3(C), was unable to precipitate Crm1 from egg extracts, consistent with a role of this sequence in mediating the Crm1 interaction (Fig. 2B).

To determine whether full-length Crk protein deficient in the candidate NES would be localized to nuclei more strongly than the wild-type protein, we transfected myc-tagged wild-type or NES(−) Crk proteins into U2OS cells and processed

them for immunoflourescence with anti-myc antibodies. As shown in Fig. 3A (with corresponding Hoechst staining of DNA shown in panel B), wild-type Crk II localized throughout the cytoplasm and nucleus. In contrast, the NES(−) mutant was largely nuclear (Fig. 3C, and corresponding Hoechst staining of DNA in panel D). These data are consistent with the identified sequence functioning as an NES and suggest that NES(−) mutant Crk entering the nucleus was retained there due to an inability to bind Crm1.

Crk II is well conserved among vertebrates, with ~80% or greater similarity among mammal, frog, and chicken homologues (12). As shown in Fig. 1A, the residues within the Crk SH3(C) that constitute the NES are virtually identical in all sequenced vertebrate Crk proteins and in the human Crk-like protein CrkL. Interestingly, despite some sequence divergence, hydrophobic residues with similar spacing in the C-terminal SH3 domain of *Drosophila* Crk might also be expected to confer binding to Crm1. However, this NES does not seem to be conserved in the Crk-like protein from *C. elegans*, CED-2, suggesting that this region of Crk may not function similarly in that organism.

A Crk-Wee1 interaction in mammalian cells is enhanced by mutation of the Crk NES. As described above, experiments with *Xenopus* egg extracts demonstrated that apoptotic induction by Crk depended upon binding to Wee1, a known nuclear constituent (42). Previous studies of Crk SH2 interactors in mammalian cells had identified several Crk binding-proteins, including an unidentified tyrosine-phosphorylated protein of ~110 kDa (for review, see reference 27). Because the Wee1 band (107 kDa) is similar in size to this band, we speculated that mammalian Crk, like its *Xenopus* counterpart, might engage the tyrosine kinase Wee1 within nuclei. Moreover, we predicted that any NES-deficient Crk [NES(−) Crk] entering the nucleus should be trapped there where it might interact with Wee1 to a greater degree than the wild-type protein. To assess this, we transfected myc-tagged wild-type or NES(−) Crk into NIH 3T3 cells and then, 24 h later, immunoprecipitated Crk with anti-myc antibody, resolved precipitates by SDS-PAGE, and immunoblotted with antiphosphotyrosine antibody. While both wild-type and NES(−) Crk proteins were immunoprecipitated at similar levels (see lower panel in Fig. 4) and both bound to a tyrosine-phosphorylated protein of 107 kDa (Fig. 4, upper panel), the interaction with the NES(−) Crk was strikingly enhanced relative to that of the wild type. Given the poor quality of all of the commercially available anti-Wee1 antibodies we tested, we elected to examine the possibility of a Wee1-Crk interaction directly by cotransfecting untagged wild-type or NES(−) Crk with myc-tagged human Wee1. As shown in Fig. 5, Wee1 coprecipitated with both Crk proteins, but again, the association with the NES(−) Crk was considerably more striking. Not surprisingly, tyrosine phosphorylation of Wee1 is required for interaction with the SH2 domain of Crk (42). Interestingly, the known site of *Xenopus* Wee1 phosphorylation that conforms to a Crk binding consensus appears to be conserved in human Wee1 (Fig. 5B). Indeed, treatment of cells with sodium orthovanadate to inhibit tyrosine phosphatase activity allowed detection of endogenous Crk-Wee1 interactions (Fig. 5C). Collectively, these data not only demonstrate, for the first time, an association between mammalian Crk and Wee1 proteins, but also highlight the idea

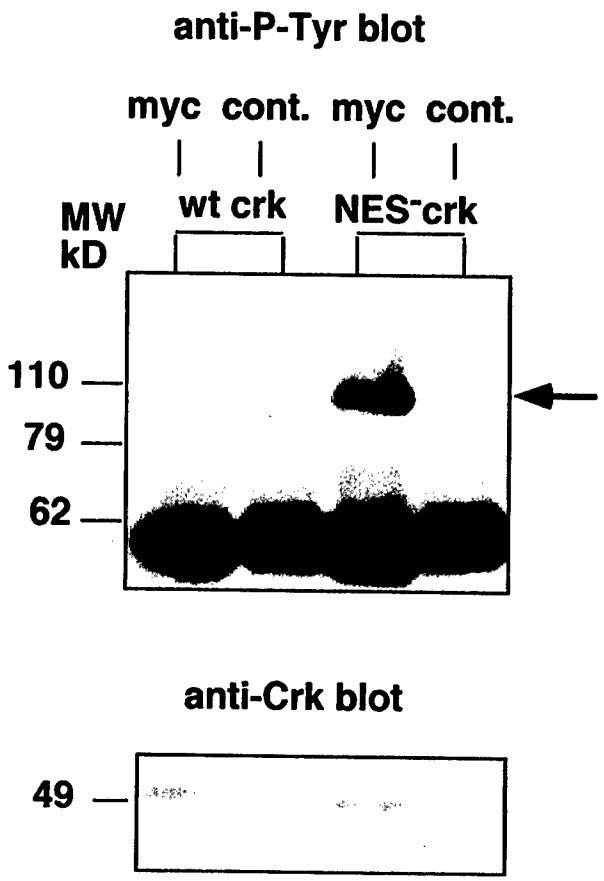

FIG. 4. A tyrosine-phosphorylated band of ~107 kDa coprecipitates with NES(−) Crk. NIH 3T3 cells were transfected with pEBB myc-wt or myc-NES(−) Crk. Cells were lysed, and lysates (~500 μg of total protein per sample) were incubated with immuno-resins consisting of anti-myc or control (cont. [anti-FLAG]) monoclonal antibodies bound to protein G-Sepharose. Immunoprecipitated material was washed three times in IP buffer to remove nonspecifically associated material. Bound proteins were eluted by boiling resins in SDS-PAGE sample buffer and detected by immunoblotting with an antiphosphotyrosine antibody (upper panel) or anti-Crk antisera [lower panel; similar amounts of wild-type and NES(−) Crk in immunoprecipitates]. MW, molecular mass (kilodaltons).

that Crk restricted to the nucleus might engage a distinct signaling pathway.

NES-deficient Crk protein induces cell death. Since Crk binding to Wee1 was required for apoptosis in *Xenopus* egg extracts, we wished to determine whether the NES(−) and wild-type Crk proteins, which differed in their ability to bind Wee1, would also display differential apoptotic activities in intact mammalian cells. Parrizas et al. (36) reported that overexpression of wild-type Crk II in 293 cells caused increased death in this cell line (36). We found that overexpression of NES(−) Crk consistently caused a three- to sevenfold increase in apoptosis compared to wild-type Crk (Fig. 6). These relative death rates were measured by the luciferase assays shown in Fig. 6 and also by scoring DNA fragmentation after Hoechst staining, as well as by counting GFP-positive surviving cells following cotransfection of GFP- and Crk-expressing plasmids (data not shown). Representative fields from a GFP cotransfecton experiment are shown in Fig. 7; cultures cotransfected

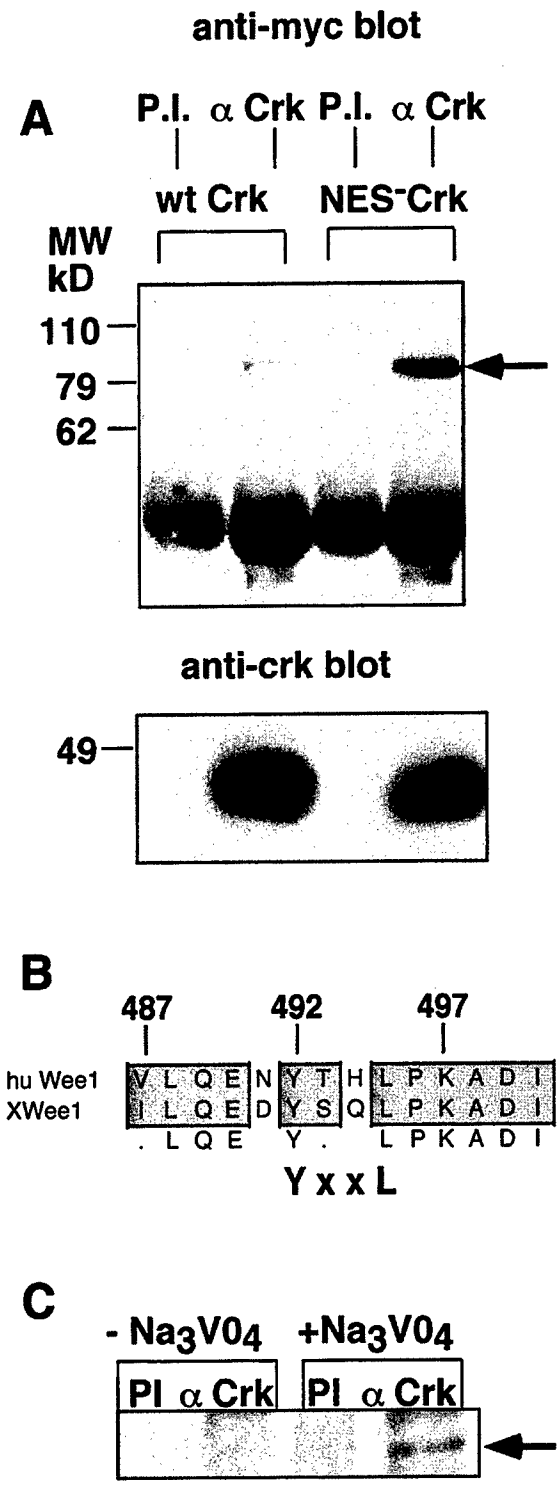

FIG. 5. Wee 1 coprecipitates with NES(−) Crk. (A) untagged pEBB-wt Crk or NES(−) Crk were cotransfected with myc-Wee1 in NIH 3T3 cells. Cells were lysed and lysates (~500 μg of total protein per sample) were incubated with immuno-resins consisting of anti-Crk antisera or preimmune sera bound to protein A-Sepharose. Nonspecifically bound material was removed by washing resins three times with IP buffer. Bound proteins were eluted by boiling resins in SDS-PAGE sample buffer and detected by immunoblotting with an anti-myc antibody (upper panel) or an anti-Crk antibody [lower panel; demonstrates similar amounts of wild-type and NES(−) Crk in immunoprecipitates]. MW, molecular mass (kilodaltons). (B) An alignment of the putative Crk SH2-binding consensus sequence (YXXP/L) in *Xenopus* (XWee1; residues 398 to 411) and human (hu Wee1; residues 487 to 500) Wee1 homologues. (C) Endogenous Wee1 coprecipitates with endogenous Crk in orthovanadate-treated NIH 3T3 cells. Indicated samples were treated with 50 μM sodium orthovanadate for 16 h. Cells were lysed, and lysates (~1 mg of total protein per sample) were incubated with immuno-resins consisting of anti-Crk or preimmune polyclonal antibodies bound to protein A-Sepharose. Immunoprecipitated proteins were washed three times in IP buffer to remove nonspecifically bound material. Bound proteins were eluted by boiling resins in SDS-PAGE sample buffer and detected by immunoblotting with an antiphosphotyrosine antibody.

with the NES-deficient Crk mutant and GFP showed markedly fewer GFP-positive cells (Fig. 7C) than those transfected with the vector plasmid (Fig. 7A) or wild-type Crk (Fig. 7B). Note that the GFP-positive cells remaining in the NES-deficient transfectant clearly exhibited an apoptotic morphology. Collectively, these data suggest that mutation of the Crk NES both enhances Wee1 binding and promotes apoptosis.

If Crk-Wee complexes are, indeed, proapoptotic, we might predict that apoptotic stimuli would enhance complex formation. Since DNA-damaging agents such as ionizing radiation can either stop the cell cycle or induce apoptosis, depending on the extent of damage, we reasoned that Wee1, participating in the regulation of both cell cycle progression and cell death, might be ideally positioned to transduce a radiation-responsive DNA damage signal. Accordingly, we transfected cells with myc-tagged Wee1 and subjected them to a strong damaging signal (20 Gy of ionizing radiation) and then analyzed anti-myc immunoprecipitates for the presence of endogenous Crk protein. As shown in Fig. 8, X-irradiation consistently produced a modest (approximately twofold) increase in Wee1-Crk complex formation, consistent with its possible involvement in signaling from DNA damage.

Localization of Crk to the cell periphery is dependent upon growth factors and Crm1-mediated nuclear export. Studies of Crk subcellular localization have placed it both in the nucleus and at the cell periphery, where it can be incorporated into either focal adhesions or in membrane ruffles at the leading edge of migrating cells. In a number of different cell lines (293, NIH 3T3, etc.), we have seen a substantial nuclear pool of wild-type Crk, both by immunofluorescence and by expression of GFP-Crk fusion proteins (Fig. 3 and 9) (data not shown). If the nuclear pool of Crk is proapoptotic, why do healthy, normal cells exhibit nuclear Crk staining? Given reports that Crk promotes cell survival either as part of focal adhesion complexes or by Cas-Crk coupling at the edge of migratory cells, we suspect that any proapoptotic signals emanating from the nuclear pool of Crk are antagonized by pro-survival signals from Crk-containing structures at the cell periphery. When the NES(−) Crk protein was overexpressed as described above, it is likely that the balance was shifted to favor cell death over cell survival. Cheresh and colleagues (8) have observed that Crk is present at the cell periphery under normal growth conditions, in rich medium (when survival signals would be expected to predominate), and leaves the periphery under starvation conditions that would, if continued, promote apoptosis. Interestingly, upon restimulation with insulin, Crk reappears at the cell periphery (23). In similar experiments, we performed immu-

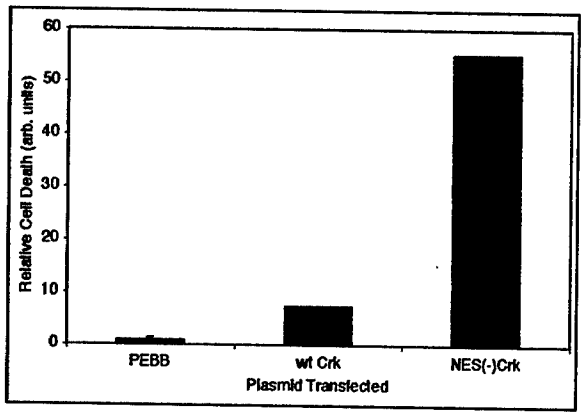

FIG. 6. Overexpression of NES(−) Crk in tissue culture cells increases apoptotic cell death. pEBB (empty vector), pEBB-wt Crk or NES(−) Crk (1.5 μg of DNA per 35-mm-diameter well) was contransfected with constitutively active luciferase reporter construct (DNA amount, 1:10). After 24 h of transfection, cells were lysed and lysates were processed for luciferase activity. The graph shows relative cell death plotted as the inverse of luciferase activity (arbitrary units).

nofluorescence with anti-Crk antibodies on cells subjected to a regimen of growth factor starvation and restimulation. However, in order to see if Crk relocalization to the cell periphery might be dependent upon Crk nuclear export, we performed the experiments in the presence and absence of the nuclear export inhibitor leptomycin B.

In NIH 3T3 cells starved of growth factors, Crk protein was clearly absent from the cell periphery, but could be seen both in the nucleus and in a cytoplasmic network surrounding the nucleus (Fig. 9A). Given the basal nuclear pool of Crk in these cells, it was difficult to judge whether leptomycin B treatment alone significantly affected the amount of total Crk in the nucleus. However, the intensity of perinuclear and cytoplasmic Crk staining was noticeably reduced by leptomycin B (Fig. 9B). Upon restimulation of the serum-starved cells with insulin or platelet-derived growth factor (PDGF), a detectable fraction of Crk moved to sites of membrane ruffling at the cell periphery, as has been described previously (Fig. 9C) (23). These data are consistent with the notion that Crk appears at the cell periphery when the cellular environment favors survival, but is absent from the periphery when the balance is tipped towards cell death. Importantly, the reappearance of Crk at the cell periphery was entirely blocked by leptomycin B (Fig. 9D), suggesting that the peripheral Crk that appears after growth factor treatment may well have derived from the nuclear pool of Crk. Moreover, as reported by Cheresh and colleagues (8), Crk at the cell periphery is required for the nuclear membrane ruffling seen after growth factor stimulation. Consistent with this, we found that leptomycin B treatment, which stopped Crk from moving to the cell periphery, markedly inhibited the appearance of membrane ruffles.

The NES-deficient form of Crk requires nuclei for its acceleration of apoptosis. In *Xenopus* egg extracts, addition of recombinant wild-type Crk produces only a mild acceleration of apoptosis relative to GST control protein (Fig. 9A) (12). However, a small, but significant and reproducible acceleration of apoptosis was seen upon addition of the NES-deficient mutant to egg extracts (Fig. 10A). If it is the nuclear population of Crk that is proapoptotic, the relatively increased potency of the NES(−) mutant in triggering apoptosis should depend upon the presence of nuclei. To address this issue, we exploited the ability of the *Xenopus* egg extract to undergo caspase activation in both the presence and absence of added nuclei. In these extracts, membrane vesicles bind to added chromatin to form enclosed nuclear structures containing nuclear pores that are fully capable of transporting macromolecules. In extracts not supplemented with chromatin and light membranes (which contribute to nuclear formation), cytoplasmic and nuclear compartments are intermixed. However, when sufficient amounts of nuclear-forming chromatin are added to these extracts, nuclear trafficking factors present in the extract allow specific nuclear accumulation of true nuclear constituents, while excluding cytoplasmic components (50). Although extracts supplemented with light membranes to form nuclei activate caspases more slowly than extracts lacking these membranes (most likely due to the presence of membrane-bound antiapoptotic bcl-2 family members), this property of the extract did not present a problem, since we could simply compare the apoptosis-inducing activities of wild-type and NES(−) Crk to determine whether the relatively more potent activity of the mutant was evident only after nuclear compartmentalization. Accordingly, we measured caspase activation in egg extracts supplemented with recombinant Crk (wild type or mutant) in the presence and absence of nuclei. In the absence of nuclei, wild-type and mutant Crk proteins induced caspase activation (measured by cleavage of the caspase substrate DEVD-pNA) with nearly identical kinetics (Fig. 10B). In contrast, the NES(−) Crk was a considerably more effective activator of caspases than the wild-type protein in the presence of nuclei (Fig. 10C). These data strongly support the notion that it is a population of Crk protein within the nuclear environment that promotes cell death.

DISCUSSION

A cell's decision to live or die is influenced by diverse cellular signaling pathways. In this report, we have presented data implicating nuclear Crk protein in the decision to apoptose. Having previously demonstrated a role for Crk-Wee1 complexes in promoting apoptosis in *Xenopus* egg extracts, we have now shown that mammalian Crk behaves similarly to *Xenopus* Crk in binding the tyrosine kinase Wee1 (42). Moreover, we find that in both egg extracts and mammalian cells, mutation of a binding site for the nuclear export factor Crm1 enhances Wee1 binding and renders Crk proapoptotic.

The C-terminal SH3 domain of Crk contains a Crm1-binding NES. Using the N-terminal SH3 domain from Crk as an affinity chromatography resin, as a bait in two-hybrid screens, or as a ligand for far-Western blots, a number of SH3-binding factors have been isolated and characterized (reviewed in references 14 and 27). However, similar techniques applied by a number of laboratories to the C-terminal SH3 domain have failed to yield any convincing SH3 ligands. The data presented here suggest that the C-terminal SH3 domain may not behave as a conventional SH3 domain in binding proline-rich substrates. Rather, we hypothesize that the second SH3 domain arose from gene duplication of a "real" SH3 domain, but no longer retains SH3 function. Rather, it serves as a binding site

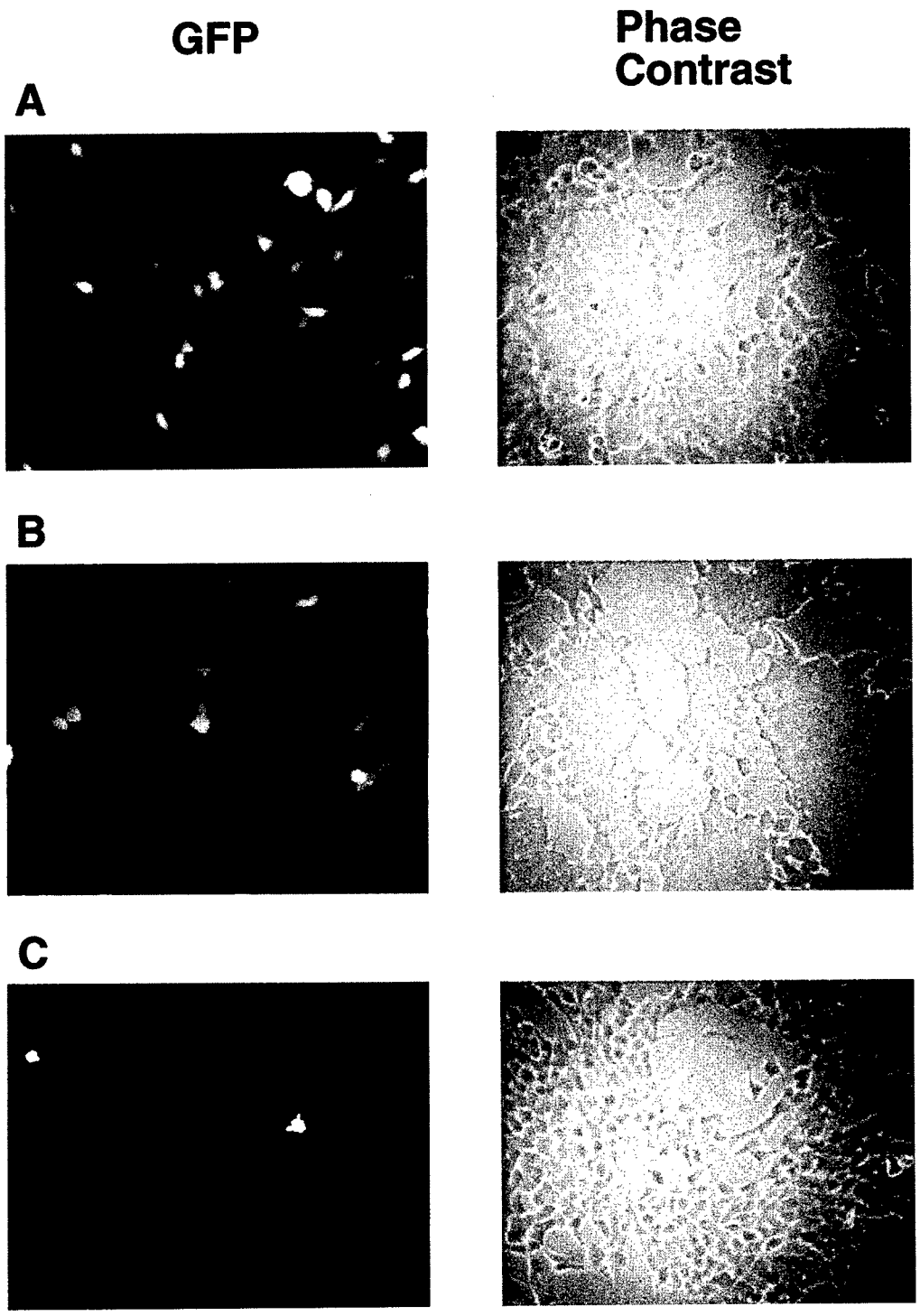

FIG. 7. Cells transfected with NES(−) Crk exhibit apoptotic cell death. pEBB (empty vector) (A), pEBB-wt Crk (B), or NES(−) Crk (C) constructs (1 μg/well) were individually cotransfected into 293 cells (20,000 cells seeded on coverslips) with a GFP reporter construct (pEGFP N2; Clontech; DNA amount, 1:10). Cells (on coverslips) were processed as described in Materials and Methods. Panels on the left display GFP-positive cells, while panels on the right demonstrate the total cell population by phase-contrast microscopy.

for the nuclear export factor, Crm1. This binding, observed both by two-hybrid screen and affinity chromatography, was abrogated by mutation of residues conserved among Crm1-binding motifs. Furthermore, introduction of this mutation into the full-length Crk protein rendered Crk mostly nuclear, consistent with this sequence functioning as an NES.

Nuclear Crk favors apoptosis. Aside from differences in subcellular localization, the wild-type Crk and its NES(−)

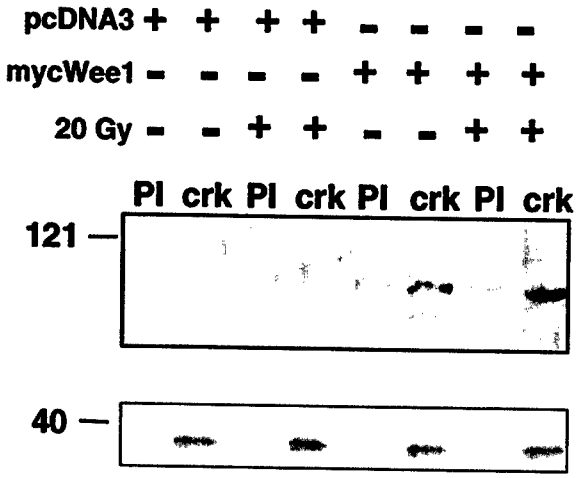

FIG. 8. Gamma irradiation enhances Crk-Wee1 association. NIH 3T3 cells were transfected with pcDNA3 (empty vector) or myc-Wee1. After 16 h, the indicated samples were mock treated or exposed to 20 Gy of gamma rays from a ^{137}Cs source. Cells were lysed, and lysates (~450 μg of total protein) were incubated with immuno-resins consisting of anti-Crk or preimmune polyclonal antibodies bound to protein A-Sepharose. Immunoprecipitated proteins were washed three times in IP buffer to remove nonspecifically bound proteins. Bound proteins were eluted by boiling samples in SDS-PAGE sample buffer and detected by immunoblotting with anti-c-myc monoclonal antibody (upper panel) and anti-Crk monoclonal antibody (lower panel).

counterpart differed in several respects: the NES mutant was considerably more efficient in binding to the tyrosine kinase Wee1 (despite equivalent cellular expression and equivalent efficiency of immunoprecipitation) and, in both *Xenopus* egg extracts and mammalian cells, the NES mutant was more potent in the induction of apoptosis. Although Parrizas et al. (36) noted enhanced apoptosis in 293 cells transfected with wild-type Crk, we found that this effect was considerably more pronounced when the NES(−) mutant was transfected. Interestingly, in *Xenopus* egg extracts, the relatively enhanced ability of the NES(−) mutant to induce caspase activation was dependent upon the presence of nuclei. These data strongly implicate the nuclear pool of Crk in the promotion of apoptosis.

Endogenous Crk protein has been observed in nuclei, in the cytoplasm, and at sites on the cell periphery (focal adhesions and membrane ruffles) (8, 9, 19, 20, 22, 23). Indeed, in every cell type we examined, endogenous Crk was both cytoplasmic and nuclear, consistent with the staining we observed when using exogenously expressed myc-tagged Crk (Fig. 3) (data not shown). Several reports have implicated Crk at the cell periphery (either in focal adhesions or in membrane ruffles) in promoting cell survival. For example, Cho and Klemke (9) demonstrated that Crk-Dock180 complexes present at the cell periphery facilitated both suppression of apoptosis and actin reorganization of cells invading an extracellular matrix (9).

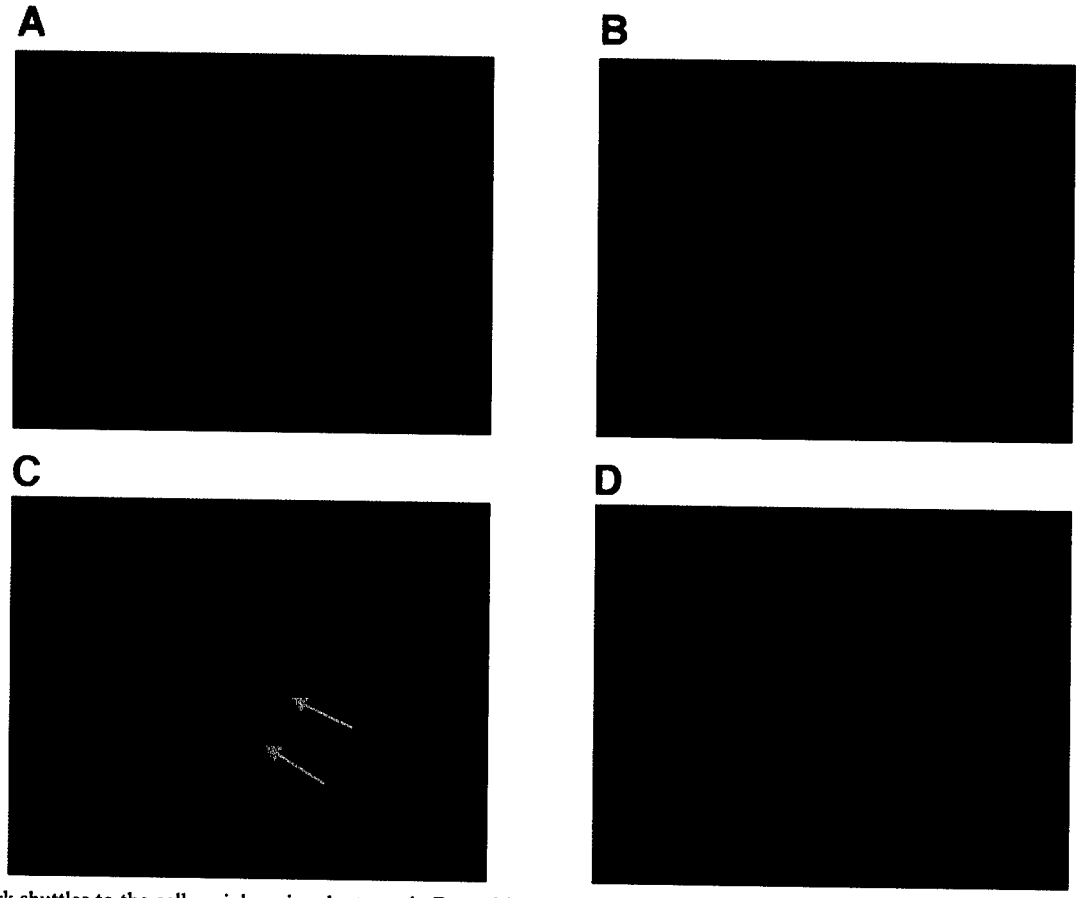

FIG. 9. Crk shuttles to the cell periphery in a leptomycin B-sensitive manner, following growth factor stimulation. NIH 3T3 cells were seeded on coverslips and subsequently serum starved for 24 h (A). Certain cell samples (B and D) were then treated with the Crm1 inhibitor leptomycin B (5 ng/ml) or vehicle (A and C) for 4 h. Cells were then refed with insulin (25 μg/ml) (C and D) or control medium (A and B) for 15 min. Coverslips were subsequently processed for immunostaining with anti-Crk antisera.

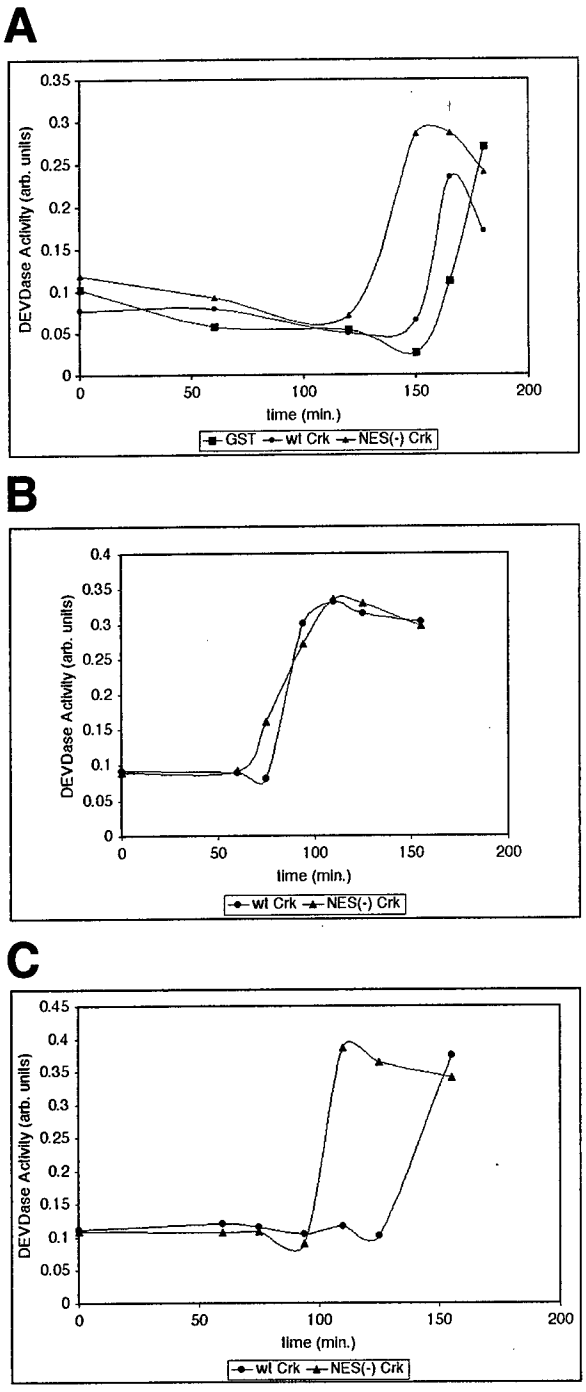

FIG. 10. Addition of NES(−) Crk to egg extracts accelerates apoptotic activity. (A) GST, wild-type Crk, or NES(−) Crk was added (500 ng/μl) to crude extracts containing exogenous nuclei (by addition of demembranated sperm chromatin); alternatively, NES(−) Crk or wild-type Crk was added (500 ng/μl) to reconstituted extracts consisting of purified cytosol (B) and mitochondria or purified cytosol, mitochondria, light membranes, and sperm chromatin (C) (to form nuclei). During a room temperature incubation, extract aliquots were taken at indicated times and processed for cleavage of the caspase substrate DEVD-pNA. DEVDase activity was measured in arbitrary units.

More recently, Stam et al. (43) have reported that the viral form of Crk, forcibly localized to the plasma membrane by virtue of its Gag domain, enhances cell survival through activation of AKT pathways. In addition, cells that lose contact with the substratum or neighboring cells are known to undergo anoikis, a type of apoptotic cell death. Because Crk is known to participate in focal adhesion signaling, this type of cell death may reflect, in part, loss of peripheral Crk-mediated survival signals (16, 17, 19, 20, 22).

The propensity of a cell to survive or die is set by the balance between proapoptotic and prosurvival signals. Bearing this in mind, we propose that Crk at the periphery acts to promote cell survival, while nuclear Crk promotes apoptosis. Consistent with this hypothesis, Cheresh and colleagues (8) have observed that Crk is present at the cell periphery under normal growth conditions, in rich medium (when survival signals would be expected to predominate), and leaves the periphery under starvation conditions that would, if continued, promote apoptosis. Interestingly, upon restimulation with insulin, Crk reappears at the cell periphery (23). In similar experiments reproducing this regimen, we found that this reappearance of Crk at the cell periphery was blocked by the Crm1 inhibitor, leptomycin B. These results are consistent with regulated Crk shuttling in response to growth factor stimulation, a possibility that merits further future investigation.

The Wee1-Crk complex and promotion of apoptosis. Given the paradigm for signaling involving adapter proteins, it is plausible that Wee1 serves either to localize Crk to other signaling molecules in a particular subnuclear locale or to allow Crk to encounter other Wee1-associated proteins. Although a proportion of the wild-type protein can be seen within nuclei, we did not observe high levels of Crk-Wee1 complexes after wild-type protein transfection. While the enhanced binding of the NES(−) mutant to Wee1 almost certainly reflects a greater nuclear accumulation of this protein, it is also possible that Crm1 binding to the C-terminal SH3 domain in the wild-type protein in some way impedes binding of the SH2 domain to Wee1. Alternatively, Crm1 binding may bring Crk protein to an intranuclear localization (e.g., proximal to the nuclear pores) inaccessible to Wee1. Careful examination of the intranuclear distribution of the wild-type and mutant Crk proteins coupled with future experiments to determine if Wee1 and Crm1 can bind Crk simultaneously should help to answer these questions.

Given the fact that Crk can only bind tyrosine-phosphorylated Wee1, we speculate that apoptotic stimuli might stimulate Wee1 tyrosine phosphorylation, thereby promoting Wee1-Crk complex formation and eventual cell death. While it is clear that all apoptotic stimuli will not trigger Wee1-Crk complex formation, we should be able to detect enhanced complex formation in response to particular apoptotic triggers. Indeed, complex formation between Wee1 and Crk was enhanced following treatment of cells with ionizing radiation. This raises the possibility that radiation (and probably other apoptotic stimuli) either stimulates a Wee1-directed tyrosine kinase or promotes Wee1 autophosphorylation. In this regard, it is interesting to note that mutation of *Xenopus* Wee1 at tyrosines 90, 103, and 110 abrogated Crk binding (42). Because Wee1 can autophosphorylate at all three of these sites in vitro, it may be that apoptotic signaling molecules can stimulate Wee1 au-

tophosphorylation. Although the nature of such a stimulatory molecule(s) is currently unclear, it is interesting to note that the Ser/Thr kinase Mos can promote Wee1 autophosphorylation in the context of the first embryonic cell cycle; this raises the possibility that a distinct Ser/Thr kinase, activated under apoptotic conditions, might also act to stimulate Wee1 autophosphorylation (31). Alternatively, apoptotically regulated Wee1-binding proteins might control its propensity to autophosphorylate. Finally, although Wee1 can autophosphorylate at sites required for Crk binding, it is certainly possible that another kinase phosphorylates those sites on Wee1 in vivo.

If Crk at the cell periphery does favor cell survival, increasing the nuclear pool of Crk could trigger cell death simply by titrating N-terminal SH3-binding factors necessary to transmit survival signals away from the cell periphery, sequestering them within nuclei. Under these circumstances, Wee1 might act simply to anchor the nuclear Crk, aiding in sequestration. However, in *Xenopus* egg extracts (which clearly lack a "periphery" of any sort), immunodepletion of either Crk or Wee1 actually prevents apoptosis, suggesting that a proapoptotic pathway employing these proteins operates in these extracts (42). While further experimentation will be necessary to distinguish between these mechanisms of Crk-Wee1 action, it will be interesting to compare the population of proteins bound to the N-terminal SH3 domain of Crk in nuclei to those bound to Crk located at the cell periphery. If a distinct proapoptotic Crk-Wee1 signaling pathway exists, we might expect to see different SH3 binders engaged in these two compartments. Indeed, one attractive candidate for transmission of a proapoptotic signal is the nuclear tyrosine kinase c-abl, a known binder of the N-terminal SH3 domain of Crk that can impinge on both apoptotic signaling and shuttle to and from the nucleus (18, 39, 44). Indeed, when the cytoplasmic oncogenic variant of abl, Bcr-Abl, is forcibly localized to the nucleus, apoptosis ensues (49). Future experiments will be aimed at examining this and other SH3 interactors to elucidate the pathway by which nuclear Crk contributes to cell death.

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